Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts

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Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts

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SUMMARY

During hindbrain development, cells become segregated into segmental groups, rhombomeres, by mechanisms that are presently unknown. One contributory factor early in development may be an alternating periodicity in cell surface properties down the neuraxis. This possibility was previously suggested by experiments in which tissue from different segmental levels was apposed in the absence of a boundary. New boundaries were regenerated only when rhombomeres from adjacent positions or positions three rhombomeres distant from one another were apposed. Combinations of two odd-numbered or two even-numbered rhombomeres usually failed to generate a boundary. In order to pursue this phenomenon to the cellular level, we have used two approaches, both involving donor-to-host transplantation. First, quail rhombomeres were grafted at various hindbrain levels of a chick host. Apposition of rhombomere 4 (r4) with r3 was concomitant with negligible cell mixing across the interface. By contrast, combinations of r3 with r5 or with r3 tissue led to cell mixing that was more extensive in combinations of identical rhombomeres (r3 with r3) than between two alternate ones (r3 with r5). Secondly, we grafted small pieces of fluorescently prelabelled chick rhombomere tissue at various hindbrain levels of chick hosts. In most cases, cells dispersed widely when transplanted orthopically or two segments distant from that of their origin. Cells transplanted into an adjacent segment, however, showed a tendency to remain undispersed. Among the different graft combinations, furthermore, there was a variation in the extent of dispersal that showed an additional level of complexity not revealed in boundary regeneration experiments. The possibility is raised that the early partitioning of rhombomeres involves a hierarchy in the adhesive preferences of cell-cell interactions along the neuraxis.

Key words: rhombomere, hindbrain, transplantation, cell mixing, chick, quail

INTRODUCTION

The process of segmentation appears to be fundamental for the development of the vertebrate hindbrain. Albeit that rhombomeres exist only transiently, they dictate the cellular ground plan that presages the emergence of local diversity in the hindbrain neuroepithelium (Lumsden, 1990). Rhombomeres delimit domains of expression of genes, such as those of the Hox clusters, that are assumed to play a role in pattern formation (reviewed in McGinnis and Krumlauf, 1992; Graham, 1992). Temporal and spatial patterns of neurogenesis accentuate the importance of the segmental plan. Neural differentiation is more advanced in even-numbered than in odd-numbered segments, and neuronal subpopulations are organised segmentally. For instance, groups of branchiomotor neurons occupy pairs of segments, their axons innervating a single branchial arch (Lumsden and Keynes, 1989). Neural crest cells bear a similar relationship to the periphery as motor axons, with crest cells emerging in segmental streams to populate single branchial arches (Lumsden et al., 1991). At later stages of development, repertoires of different neuronal types constitute variations on a segmental theme (Clarke and Lumsden, 1993), though segmentation of neuronal groups is soon obscured by the migration of neuronal somata and rearrangment of axons, accompanied by the eventual regression of rhombomere boundaries at around stage 25 (Hamburger and Hamilton, 1951).

Rhombomere boundaries appear in a stereotyped sequence between stage 9– and stage 12 (Vaage, 1969). At around the time boundaries first become visible, the normal interkinetic nuclear migration characteristic of the neuroepithelium is reduced only at the boundaries; nuclei tend to be retained close to the ventricular surface, creating a region of low cell density beneath (Lumsden and Keynes, 1989; Guthrie et al., 1991). These basal regions later become prominent conduits for axonal outgrowth, with axons accumulating precociously from the marginal zone towards the ventricular surface. Boundaries also possess a number of specialised features that become refined during later developmental stages; they express enhanced levels of laminin, Ng-CAM/L1 (Lumsden and Keynes, 1989) and filamentous actin (Guthrie et al., 1991), and bind peanut lectin (Layer and Alber, 1990).

Rhombomeric territories appear to be separated into non-mixing cell groups before or at the time of boundary formation. When single progenitor cells were marked prior to boundary formation, labelled cells mingled with unlabelled cells and clones frequently spanned more than one segment. When cells were marked at or after the time of boundary appearance, however, the resulting clone was restricted to a single segment (Fraser et al., 1990). Thus, from the time when boundaries are first morphologically recognisable, rhombomeres are compartments similar to those of insects (Garcia-Bellido et al., 1973). How do these cell groups become segregated? The conspicuousness of late stage boundaries coupled with the distinct character of individual rhombomeres suggests that boundaries might act physically to separate the cells of adjacent territories. However, there is no evidence to support this view. Rather, it seems more likely that adjacent rhombomeres first possess different identities, embodied in their differential miscibilities, in turn leading to the generation of boundary characteristics at their interfaces.

Rhombomere identity may start to be defined in terms of Hox gene expression shortly before boundary formation. When the presumptive rhombomere 4 (r4) is transplanted to an ectopic location, *Hoxb-1* (*Hox 2.9*) expression is upregulated appropriately and motor nerve morphology remains characteristic of the grafted region, despite the fact that at the earliest time of transplantation (stage 9–), the segment is not yet defined by boundaries (Guthrie et al., 1992).

Other data also emphasise the idea that rhombomeric differences are established early and are a cause rather than a consequence of boundary formation. Boundary ablation experiments showed that a new boundary could regenerate between adjacent segments within several hours of removal (Guthrie and Lumsden, 1991). Adjacent segments evidently manifest dissimilar properties, enabling them to recognise and restore a deficit when a band of cells is removed. This experiment tested only the regeneration potential of tissue from segments that would normally lie adjacent to one another. Donor-to-host transplantation experiments then evaluated the effect of confronting rhombomeres from different positions in the absence of boundary cells. Apposition of adjacent rhombomeres or those three segments distant from one another led to boundary reconstruction. Conversely, confrontation of identical rhombomeres, or of two different odd-numbered or even-numbered rhombomeres failed to give rise to a new boundary in the majority of cases. This suggested an alternating segmental periodicity of cell surface properties along the neuraxis.

An alternating cell state seems a plausible and parsimonious mechanism for generating a segmental pattern (Lumsden, 1990). An expected consequence of such cell surface properties might be that cells from different rhombomeres would exhibit selective adhesion. In our previous study (Guthrie and Lumsden, 1991), we examined neither the ability of cells from different rhombomeres to mix with each other, nor the correlation with boundary formation. However, we hypothesised that failure of cells from adjacent rhombomeres to mix would lead to a sharp interface between the two cell populations and eventual reconstruction of a boundary. Conversely, the confrontation of cells from duplicate rhombomeres, or from two even-numbered or odd-numbered rhombomeres would allow cell mixing, in the absence of a discrete interface, with the concomitant failure to regenerate a boundary.

In this study, we have investigated the extent of mixing or segregation exhibited by cells from different rhombomeres using two complementary experimental methods. In the first approach, we have confronted rhombomeres of different positional origin using transplantation of quail tissue into a chick host. Grafted embryos were examined two days later for the presence or absence of a boundary and quail cells in this region were recognised by their Feulgen-positive nucleoli. Mapping of labelled and unlabelled cells at the interface in various transplant combinations then showed the extent of mixing between different populations. The second approach involved transplantation of very small pieces of individual chick rhombomeres into chick hosts at various hindbrain axial levels. Tissue fragments were labelled with a nuclear dye that enabled graft and host to be clearly distinguished down to the single cell level. Embryos were analysed two days later, after the grafted cells had had the opportunity to mix with or remain segregated from the unlabelled host cells in their vicinity. While heterospecific grafts explored cell behaviour at a single interface perpendicular to the midline, homospecific grafts allowed cells from a small plug of tissue to confront the host environment on several sides. In addition to allowing us to evaluate our hypothesis about the alternate periodicity of cell miscibility, it was hoped that these experimental paradigms might reveal more subtle differences in the behaviour of rhombomeric populations than had been deduced from simple assessment of boundary regeneration.

MATERIALS AND METHODS

Rhode Island Red hens' eggs and Japanese quails' eggs were incubated to chick stages 9-12 (Hamburger and Hamilton, 1951), and to the equivalent stages for quails. Eggs were then windowed and embryos made visible by sub-blastodermal injection of India ink. Embryos used as donors were removed from the eggs at this stage. Microsurgery was performed through a small opening in the vitelline membrane using needles, flame-sharpened from 100 μm diameter pure tungsten wire.

Transplantation of rhombomeres (quail to chick)

Rhombomere transplantation experiments were performed in order to confront rhombomere tissue from different axial levels in the absence of boundary cells (for details see Guthrie and Lumsden, 1991). The main departure from the previous method was that quail embryos were used as donors, matching them to chick hosts of the same stage by somite count. At the stages used, the size difference between quail and chick rhombomeres is negligible. Only three experimental combinations were essayed; quail r3 placed caudal to chick r3 (r3:3 grafts), quail r4 placed caudal to chick r3 (r3:4 grafts), and quail r5 placed caudal to chick r3 (r3:5 grafts) (Fig. 1A-C). Grafts were unilateral, which is less disruptive of normal morphogenesis and leaves one side as a control. Operated eggs were sealed with tape and returned to the incubator for 36-48 hours. After this time, most embryos had reached stage 19-20, and only those where grafts had healed adequately (about 40%) were analysed further. These embryos were fixed in a potassium dichromate, formaldehyde, acetic acid mixture (10:2:5 parts), dehydrated and embedded in paraffin wax. They were then sectioned parasagit-

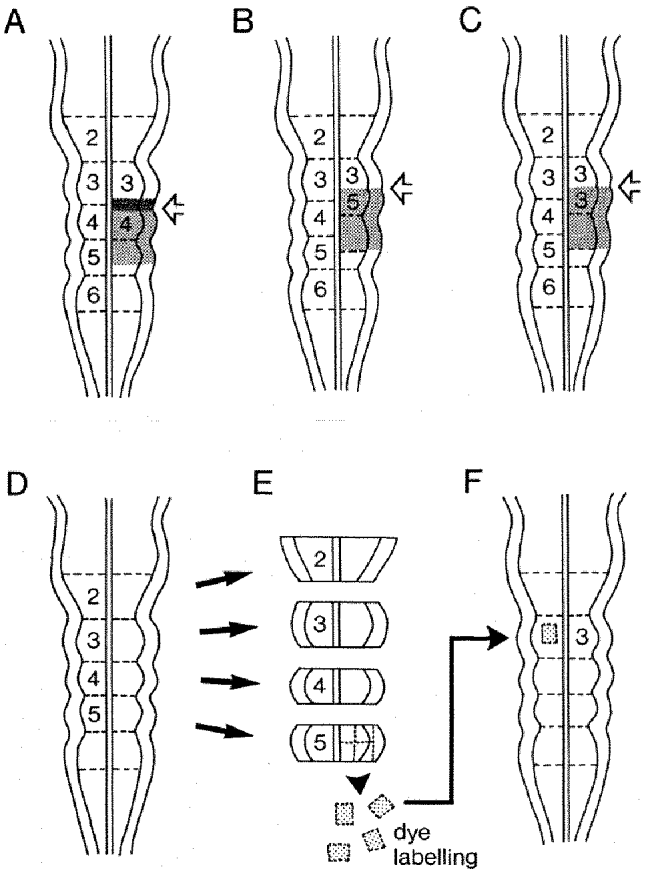


Fig. 1. Diagrammatic summary of methods used for orthotopic and heterotopic grafting experiments. (A-C) Quail to chick rhombomere grafts. (A) r3:4 graft, (B) r3:5 graft, (C) r3:3 graft. Diagrams show stage 10-12 chick host hindbrains with rhombomere boundaries indicated by dotted lines. Grafts of quail tissue were made unilaterally, so that cells from different rhombomeres come into apposition in the absence of boundary tissue. Shaded areas represent quail tissue, unshaded areas represent chick tissue, with open arrows showing the position of junction between the two. Heavily shaded band in A shows position of new boundary that forms in the r3:4 graft, but not in the other two graft combinations. (D-F) Chick to chick grafting of rhombomere pieces. (D) Stage 10-12 donor hindbrain is cut into rhombomeres, conserving r2, r3, r4 and r5. (E) Individual rhombomeres are cut into smaller pieces, and fluorescently labelled (see text). (F) A single rhombomere piece (r5 level) is transplanted heterotopically into a host brain (r3 level).

tally at 6 μm and the sections processed according to the Feulgen-Rossenbeck procedure (Feulgen and Rossenbeck, 1924).

Quantitation of cell mixing in quail/chick chimaeras

Parasagittal sections through the operated area were examined for presence or absence of a boundary, when compared with adjacent unoperated boundaries. The mode of quantitation was the same in each case. A graticule was placed over this area and cells were counted in five 7.5 μm strips of neuroepithelium on either side of a vertical line judged to demarcate the transition between territory that contained mostly chick cells and territory that contained mostly quail cells. Thus cells lying within ten vertical strips of neuroepithelium were counted, giving a figure that could be related to a particular position along the neuroepithelium. Both chick and quail cells were counted at each of these positions. Quail cells were distinguishable from chick cells by their Feulgen-positive interphase nucleolus, although the intensity of staining of different embryos varied, and it is likely that both false negatives (quail cells that stained poorly, or were not sectioned through the nucleolus) and false positives (intensely stained chick nuclei) were recorded. For each embryo, six different sections taken through various mediolateral positions were assessed. The mean number of chick and quail cells at each position along the neuroepithelium was then calculated. Thus, for each position, the number of quail cells could be expressed as a percentage of the total number of cells in a neuroepithelial strip of given area. This allowed the construction of curves showing how the percentage of quail cells altered with position from rostral (100% chick cells) to caudal (100% quail cells). Thus we could compare different embryos, despite differences in neuroepithelial thickness and in the number of cells counted per unit area.

Transplantation of rhombomere fragments (chick to chick)

The hindbrain region including surrounding tissue was dissected from donor embryos and then the mesenchyme was stripped off by incubation of brains in Dispase (Boehringer Mannheim Biochemicals Grade 1; 1 mg/ml in L15 medium (Gibco), 15 minutes at room temperature). For ease of transplanting small pieces, these grafts contained no mesenchyme, in contrast to the chick/quail grafts. Hindbrains were then subdivided into single rhombomeres (r2, r3, r4 and r5) using tungsten needles. Experiments to date suggest that, at these developmental stages, rhombomere identity is autonomous and is not influenced by mesenchymal environment (Guthrie et al., 1992; Kuratani and Eichele, 1993). Rhombomeres were incubated with the nuclear stain Hoechst 33258 (Sigma) at 50 μg/ml in Howard's Ringer for 30 minutes at room temperature, and then washed 8 times in L15. Hoechst provided a more reliable way of labelling every cell in the graft than other compounds eg. DiI. The tissue pieces used for transplantation were dissected from the single rhombomeres using needles, omitting both the dorsal-most part (neural crest) and the ventral-most part (floor plate), in view of the specialised properties of both these regions. Grafts were approximately 1/5 to 1/4 the size of a rhombomere and contained in the region of 125 cells.

Host embryos were prepared *in ovo* by aspiration with a micropipette of a small unilateral area of tissue within a single rhombomere, usually just lateral to the floor plate/basal plate boundary. Donor pieces were transferred into eggs by pipette and manoeuvred into place using needles, maintaining apical/basal polarity but neither anteroposterior nor mediolateral orientation, which was thus random. The combinations essayed were donor r2, r3, r4 or r5 into a rhombomere at the same axial level (ie. self with self), r3 into r5 and vice versa (odd-numbered combinations), r2 into r4 and vice versa (even-numbered combinations), r2 into r3 and vice versa, and r3 into r4 and vice versa (adjacent rhombomere combinations). Operated embryos were incubated for a further 36-48 hours and then hindbrains were dissected out and flat-mounted for observation with u.v. epifluorescence microscopy. The distribution of Hoechst-labelled cells was recorded in diagrams and photographs. In some cases, the precise size and shape of the graft together with the distribution and positions of fluorescent nuclei that had dispersed from it were recorded using a graticule. The statistical significance of the difference between graft categories was evaluated using the Kruskal-Wallis one-way analysis of variance (Kruskal and Wallis, 1952).

RESULTS

Transplantation of rhombomeres

In quail/chick chimaeras, transplants healed in a short time

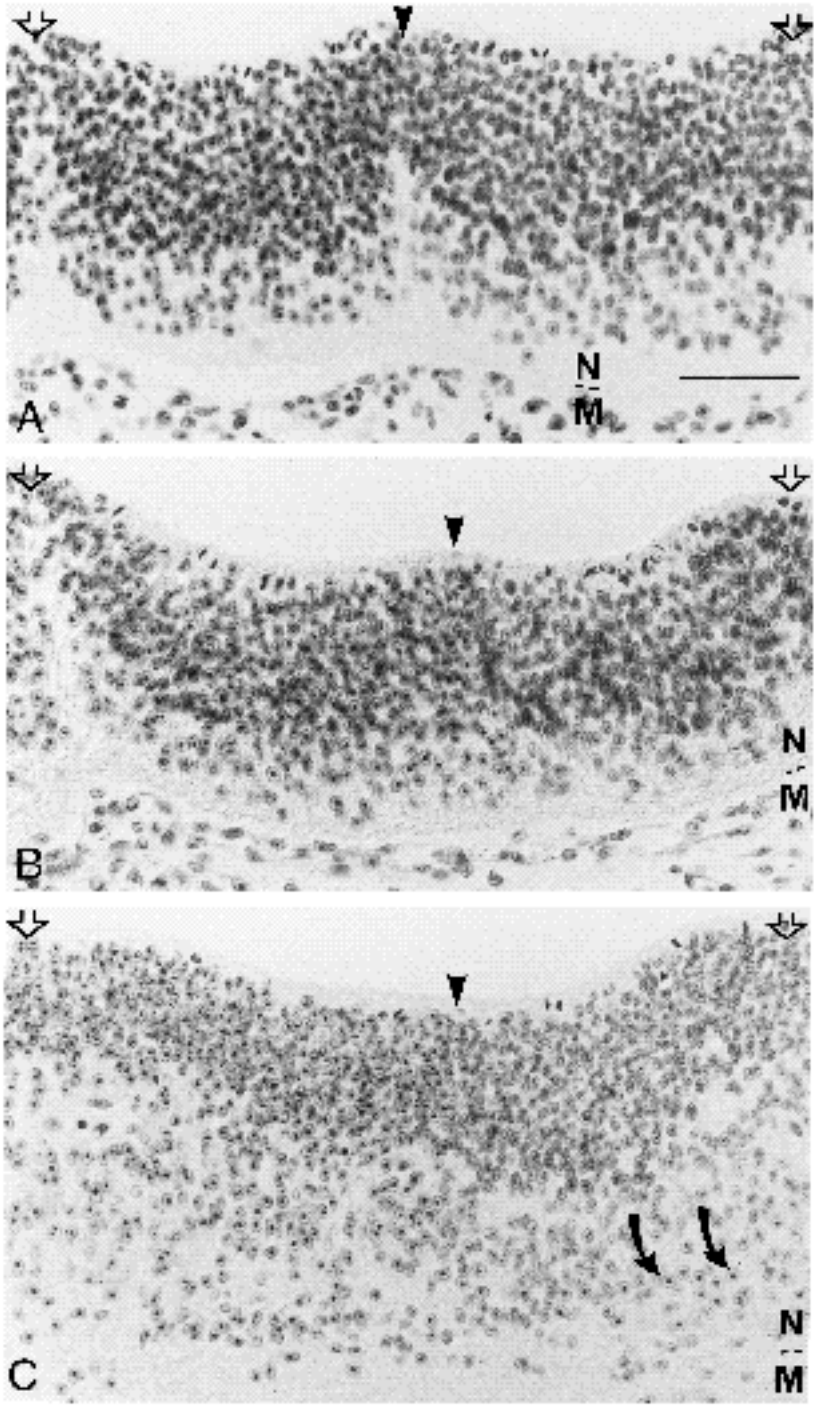


Fig. 2. Parasagittal sections of hindbrains in chick/quail chimaeras. In each case, the profile of the host/graft interface in the neuroepithelium of stage 19-21 embryos is shown. The fourth ventricle is at the the top and the mesenchyme is at the bottom (N, neuroepithelium, M, mesenchyme). Open black arrows indicate rhombomere boundaries, and black arrowheads the location of the junction between quail and chick tissue at the ventricular surface. In each case, rostral (chick tissue) is to the right, and caudal (quail tissue) to the left. (A) r3:4 graft : There is normal morphology of the reconstructed boundary (arrowhead) compared with the unoperated boundaries on either side. (B) r3:5 graft : Apart from a slight irregularity at the graft interface, the neuroepithelium is normal. At the interface there is no boundary embrasure, and there has been some limited cell mixing towards the pial side of the neuroepithelium. (C) r3:3 graft : Despite the retention of a sharp interface at the ventricular surface, an extensive amount of cell movement has taken place in deeper layers of the neuroepithelium. Quail nuclei (curved arrows) are seen occupying rostral positions in the segment, extending as far as the r2/3 boundary. Scale bar, 50 μm.

and embryos grew in an identical fashion to unoperated chick embryos. There was no significant difference in hindbrain morphology compared with previous experiments (Guthrie and Lumsden, 1991) and the number of grafts suitable for analysis was approximately 40%. Sections of ten embryos in each category, r3:3, r3:4 and r3:5 were analysed. In all ten r3:4 combinations, a morphological boundary formed an interface between chick and quail cells. In Feulgen-stained specimens, the marginal zone appears white, since it is full of axons and almost devoid of nuclei, with axons piling up in an inverted V from the marginal zone towards the ventricular surface of the boundary. This embrasure was apparent in the regenerated boundaries. At the ventricular side, boundaries also showed the characteristic convexity (Fig. 2A). By contrast, in r3:3 or r3:5 graft combinations, a boundary was never formed, and an axon-rich region was absent (Fig. 2B,C). These results are broadly consistent with the morphologies observed in chick-to-chick grafting experiments of the same rhombomere combinations.

The presence of a morphological boundary in r3:4 grafts was accompanied by an almost complete absence of cell

mixing at the interface. Often the two cell populations appeared completely separated by the apically tapering axonal zone, except immediately adjacent to the ventricular surface. Despite this fact, where a few cells were seen to have penetrated into the inapproriate territory, they were usually located close to the pial surface. Conversely, the absence of a morphological boundary in r3:5 and r3:3 embryos was associated with considerable cell mixing. This mixing was often more apparent in the pial (basal) regions of the neuroepithelium, than in the ventricular zone.

The variation in extent of cell mixing found with different graft combinations is reflected in the gradients of plots obtained by quantitating the percentage of quail cells at various positions along the neuroepithelium (Fig. 3). For clarity, only the percentage of quail cells is presented here, but it should be stressed that both chick and quail cells were quantified. The extent of movement of chick cells is implicit in the plots, with chick cells represented by the area above the curve, and quail cells represented by the area below the curve. Based on these plots there is little reason to suspect a difference in the invasiveness of chick or quail cells.

For r3:4 grafts, the transition from chick to quail cells is very sudden as illustrated by the steep gradients of the plots (Fig. 3A), which show very little variation between individual embryos. The greater degree of cell mixing observed with r3:3 and r3:5 grafts is reflected in shallower gradients for the equivalent plots (Fig. 3B,C). There was also significantly more variability in the gradients obtained for r3:3 and r3:5 embryos. Considering individual r3:5 embryos, for example, Fig. 3C (viii) resembles an r3:4 graft in the steep gradient of the transition, whereas Fig. 3C (ii), (vii) and (x) show a more gradual change. While these features were superficially similar in r3:3 grafts, there was in fact an even greater degree of cell mixing in this combination (Figs 2C, 3B). In some sections, a trail of quail cells, largely confined to the mantle zone was seen extending from the edge of the graft up to the r2/3 boundary. Thus, while the gradient of the transition zone was shallower, the distance moved by the furthest quail cells into the chick tissue was greater in r3:3 than in r3:5 grafts. A similar penetration of chick cells into quail territory occurred but was less prominent due to the invisibility of unlabelled cells against a labelled background, and the unreliability of their identification when the nucleolus is not within the section.

To quantitate the differences between the various graft types more accurately, we measured the steepest gradient between two points in the transition zone, for all of the embryos analysed. The mean gradient for r3:4 grafts was 80%, or 11% change per μm, while that for r3:5 grafts was 42%, or 6% change per μm. The mean gradient for r3:3 grafts was little different from this, 40.5% or 5.4% change per μm. Thus the rate of change of gradient for r3:5 and r3:3 grafts was roughly half that in r3:4 grafts, reflecting a dramatic difference in the degree of cell mixing. Since this index takes account of only the central part of the transition zone, where the cell mixing is changing most abruptly, it does not completely describe the situation. Another interesting parameter is thus the distance the quail cells have moved from the edge of the grafted piece into the chick tissue. Our graphs show that we could seldom record 0% or 100% quail or chick cells, due to the limitations of the marking technique, making it hard unequivocally to define the edge of the graft. Instead, we decided to measure the distance along the abscissa which defined the transition from 20% quail cells to 80% quail cells, in order to gain some impression of the mean distance being moved by quail cells into the chick host. The mean width of this 20-80% zone was only 5.9 μm in r3:4 grafts, compared with 12.8 μm in r3:5 grafts, and 19.7 μm in r3:3 grafts. Thus, this index reveals a striking difference between all three graft categories, showing that quail cells in r3:5 combinations moved roughly twice as far as those in r3:4 combinations, while cells in r3:3 combinations dispersed 1.5 times as far again as those in r3:5 grafts. The difference is even more pronounced when considering the individual embryos in each category that show the widest zone of mixing by the 20-80% criteria. On this basis, in r3:3 (Fig. 3B (iii)) cells moved at least 34.5 μm into the host tissue, more than four times as far as the 8.25 μm maximum seen in a r3:4 graft (Fig. 3A (ii)).

These results suggest a strong correlation between boundary formation and lack of cell mixing at the interface. However, they also raise the possibility that additional levels of miscibility may exist that give rise to overtly similar morphologies in terms of boundary formation.

Transplantation of rhombomere fragments

Small pieces of hindbrain neuroepithelium grafted at various rhombomeric levels generally healed in place and yielded brains with apparently normal morphology. About 16% of embryos died or were unanalysable for other reasons, while in 7% no graft was evident when viewed under fluorescence optics, suggesting that the graft had been extruded, as was sometimes reflected in the presence of one or two fluorescent cells. Preliminary experiments had shown that extensive washing of the tissue pieces eliminated any transfer of the label from donor to host. Moreover, after two days incubation of operated embryos, the marked donor cells remained brightly fluorescent, and single nuclei could be discerned against an unlabelled background. By focussing down through the specimen, it was evident that labelled nuclei lay at all levels in the neuroepithelium, suggesting that cells were undergoing a normal pattern of interkinetic nuclear migration. Some mitotic figures were present at the ventricular surface, indicating that the cells were viable, and that cell division was occurring as normal. Within a 36 hour-incubation period (3-4 rounds of cell division) this would lead to the initial 125 cells of the graft multiplying to approximately 1000-2000 cells.

The tendency of grafted tissue in each embryo to disperse or to remain as a coherent cluster of cells was recorded by allotting each embryo to one of four categories; (1) – –, a coherent cell group with fewer than 10 cells moving away, (2) –, 10-20 cells moving away from the graft, (3) +, moderate amount of dispersion of the labelled graft, and (4) ++, extensive dispersion of the graft. Categories 1 and 2 were broadly interpreted as representing absence of cell mixing, and categories 3 and 4 presence of cell mixing. All specimens were judged blind by two observers independently, though apportioning of specimens to one category or another was occasionally difficult. In some examples of dispersal, the grafted area was clearly delineated, with areas

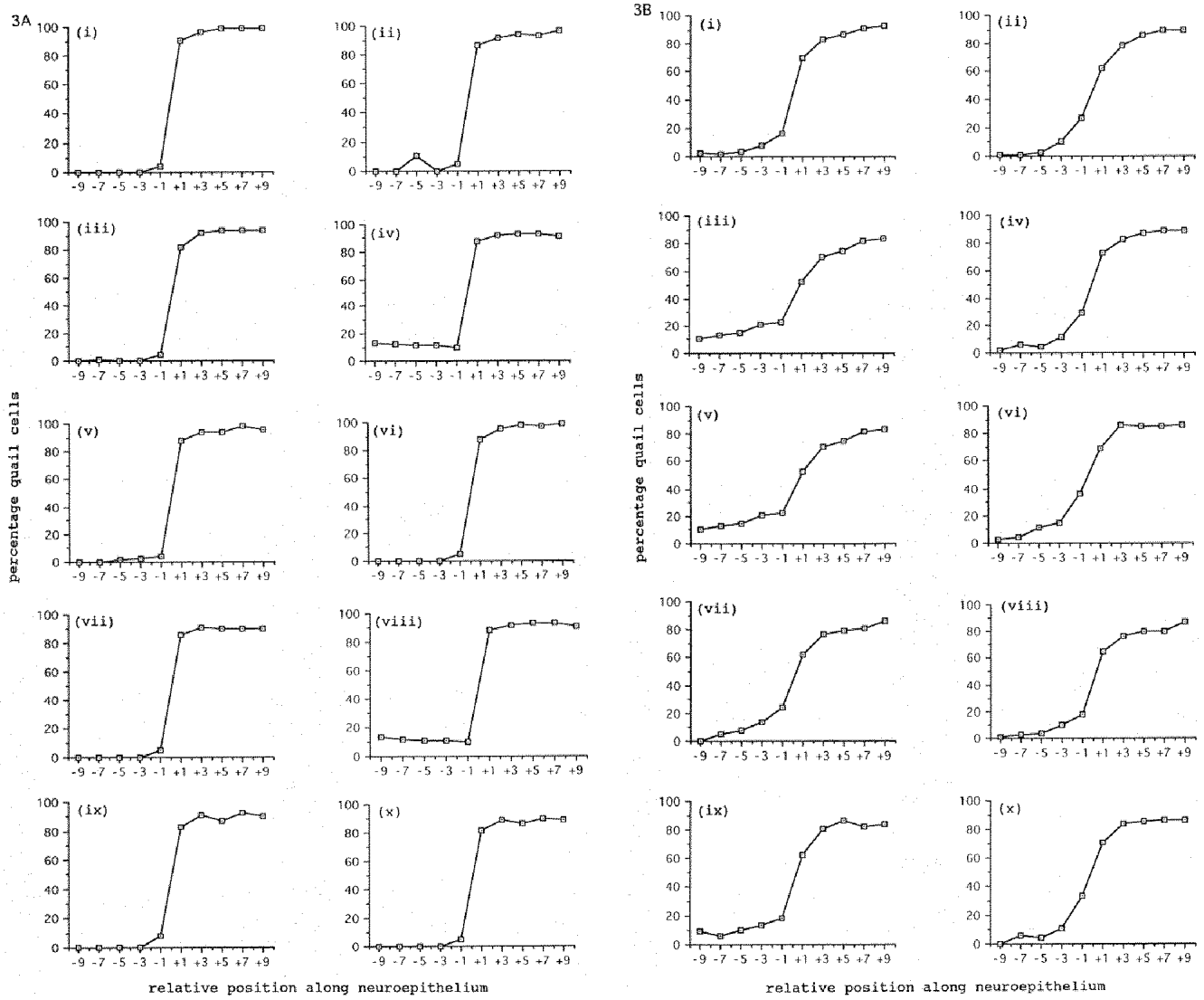
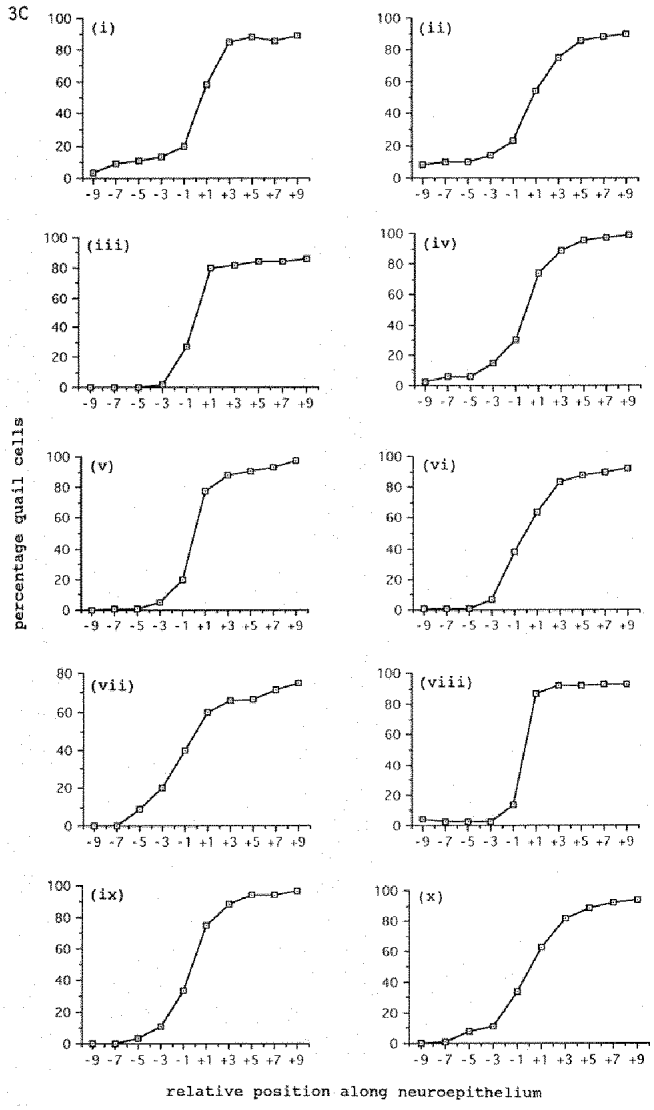


Fig. 3. Plots representing the transition zone between quail and chick tissue in grafted embryos. Each graph represents the graft interface in the hindbrain of a single embryo, with rostral (chick cells) to the left and caudal (quail cells) to the right. Abscissa shows relative position along the neuroepithelium in arbitrary units, such that 0 = the midpoint between host and graft tissue, and 1 unit = 3.25 μm. Ordinate shows the number of quail cells as a percentage of the total number of cells counted at each position (see methods). (A) i-x. r3:4 grafted embryos. (B) i-x. r3:3 grafted embryos. (C) i-x. r3:5 grafted embryos.

of lower labelling density around it into which cells had moved. In others, the graft appeared to have expanded and intermingled with the adjacent cells in all directions, rendering its edges indistinct. In embryos where the grafted piece was included in two adjacent rhombomeres, we scored it twice based on the cell behaviour in each segment.

Our broad prediction based on previous work was that self/self, odd/odd and even/even rhombomere grafts would exhibit a higher degree of cell dispersal than combinations of adjacent rhombomeres. The results from all the experiments are expressed in Table 1A. Striking differences were noted in the frequency of cell mixing (+ or ++) compared with (– or – –) depending on the level of the donor and host segment combination. Orthotopic (self with self) grafts all showed a predisposition for mixing ranging from 6/6 cases for r4 into r4, to 5/8 cases for r3 into r3. Odd into odd grafts also showed a predominance of cell mixing, 6/8 cases for r5 into r3, and 8/12 cases for r3 into r5. Comparison of even into even graft combinations r4 into r2 and r2 into r4 suggested non-reciprocity of graft combinations; while the former showed 7/7 grafts mixing, only 6/10 grafts in the latter category showed mixing. Adjacent rhombomere combinations, r2 into r3 and r3 into r2 showed that in 5/6 and 4/5 embryos, respectively, grafts failed to disperse. The adjacent rhombomere combination of r4 into r3 also showed little mixing (5/7 cases did not disperse). The only real anomaly existed in r3 into r4 combinations, in which 6/9 embryos showed dispersal.

An alternative view of the data is provided by a cell dispersal index, allowing collation of the results for each graft type. Scores for each embryo were added, based on 1 point for a – result, 2 points for – –, 3 points for + and 4



points for ++. The aggregate scores for each graft type were then divided by the number of embryos in each group, giving a score in the range 1-4 from minimal to maximal cell dispersal. The scores are shown in Table 1B. Self to self, odd/odd and even/even grafts all lie in the range 2.7-3.6, with the highest score for grafts r4 into r2 and r4 into r4. Adjacent rhombomere grafts score in the range 1.8-1.9, the exception being the anomalous category r3 into r4 which has a higher score of 2.55. It is interesting that all of the self/self grafts show a higher index than the odd/odd graft combinations, paralleling the increased dispersal in quail/chick r3:3 combinations compared with r3:5 grafts.

In order to test the statistical significance of the results obtained, we employed the Kruskal-Wallis one-way analysis of variance by ranks (Kruskal and Wallis, 1952). This is a way of determining whether differences between results for particular graft categories signify genuine population differences or represent merely chance variations such as might be expected among several random samples from the same population. To perform the test we combined the graft results into three categories. These were self/self (r2 into r2, r3 into r3, r4 into r4, r5 into r5), alternate segments (r2 into r4, r4 into r2, r3 into r5, r5 into r3) and adjacent (odd/even) segments (r2 into r3, r3 into r2, r3 into r4, r4 into r3). We then tested the null hypothesis that there is *no* difference in

Table 1. Frequency of graft dispersal in orthotopic and heterotopic chick to chick grafts of rhombomere fragments

(A) Frequency of graft dispersal in individual embryos

R2 into R2	R2 into R3	R2 into R4	
– –	– –	– –	
+	– –	– –	
++	–	– –	
++	–	–	
++	–	+	
	+	+	
		++	
		++	
		++	
		++	
R3 into R2	**R3 into R3**	**R3 into R4**	**R3 into R5**
– –	–	–	–
– –	–	–	–
–	–	–	–
–	+	+	–
+	+	+	+
	++	+	+
	++	+	+
	++	++	+
		++	+
			++
			++
			++
R4 into R2	**R4 into R3**	**R4 into R4**	
+	– –	+	
+	– –	+	
+	– –	+	
++	–	+	
++	–	++	
++	+	++	
++	+		
	R5 into R3		**R5 into R5**
	–		– –
	–		–
	+		+
	+		+
	+		+
	+		++
	++		++
	++		++

Rows show donor rhombomeres (donor r2 at top, donor r5 at bottom) and columns show host rhombomeres (host r2 at left, host r5 at right). Below each graft combination, column of symbols shows results from individual embryos, ranked from least dispersal (– –) to greatest dispersal (++).

(B) Aggregate dispersal index for each graft type

	Host			
Donor	R2	R3	R4	R5
R2	3.2	1.8	2.7	
R3	1.8	3	2.55	2.9
R4	3.6	1.9	3.3	
R5		2.75		3

Dispersal indices between 1 and 4 were calculated for each graft type (see Materials and Methods). Rows show donor rhombomeres and columns show hosts. The indices for graft types that lie on a diagonal within the table, eg. r2 into r2, r3 into r3, r4 into r4, and r5 into r5 tend to have similar values.

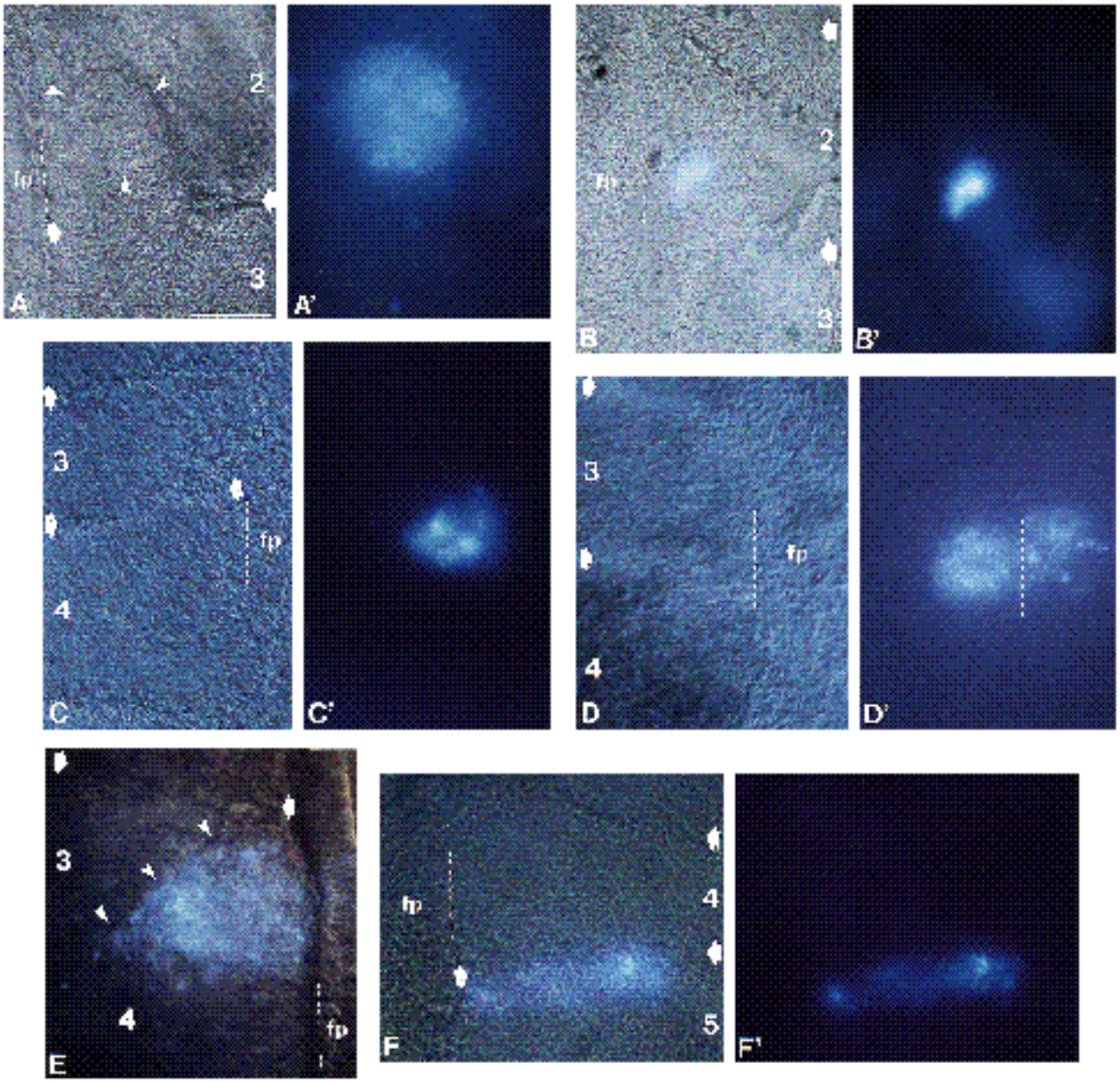
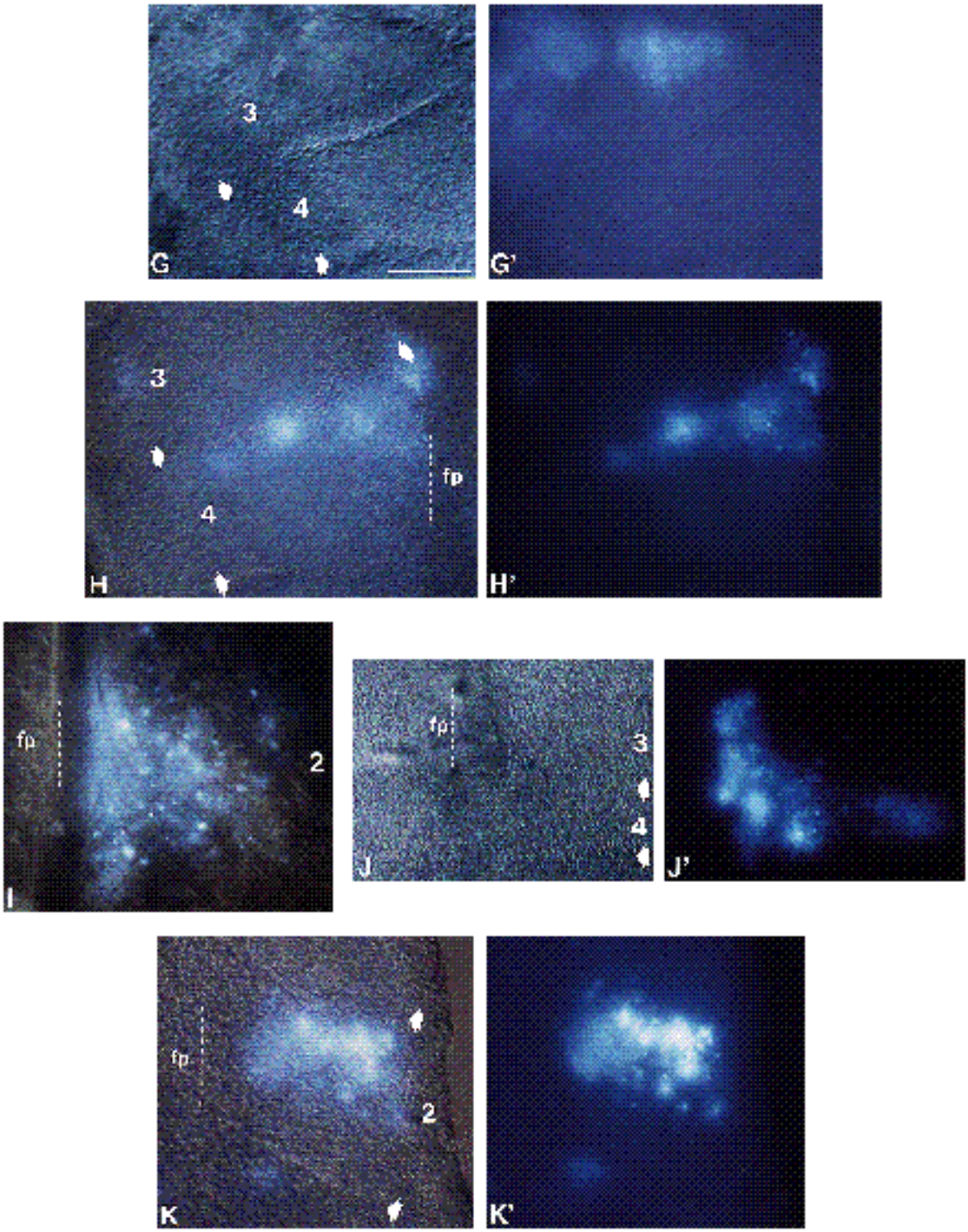


Fig. 4. Flat-mounted hindbrains containing orthotopically or heterotopically grafted rhombomere fragments. Flat-mounted hindbrains containing Hoechst-labelled rhombomere fragments were photographed using either DIC (A,C,D,G and J), epifluorescence (A',B',C',D',F',G',H', J' and K') or both (B,E,F,H,I and K). In all cases excepting E and I, paired exposures are shown for the same embryo. In the left hand panel of each pair, white arrows show the rhombomere boundaries in the vicinity of the graft. Rhombomeres are numbered and the position of the floor plate (fp) is shown by a dotted line. Right-hand epifluorescent pictures show the position of the graft in relation to these features, and the extent of dispersal of fluorescently labelled nuclei. In some cases, the nuclei of individual cells are discernable (eg. I and K'). Blurring of fluorescence is due to scattering from nuclei at various levels within the neuroepithelium. Scale bar, 110 μm in all photographs apart from A - scale bar, 160 μm, and J,J' - scale bar, 220 μm. Parts of figure are listed specifying type of graft and result category into which it fell ie. – –, –, +, or ++. (A,A') r3 into r2 (– –). White arrowheads (A) show position of boundary formed round graft. (B,B') r3 into r2 (– –). (C,C') r3 into r4 (–). (D,D') r4 into r3 (–). Part of the graft is in the floor plate. (E) r2 into r3/r4 (–/+). Graft lies partly in r3 and partly in r4 territory. A boundary has formed between the r2 cells and the host r3, making a diagonal with the host r2/3 boundary, while r2 cells have dispersed into the r4 tissue. (F,F') r3 into r5 (+). Grafted cells have spread out along the r4/5 boundary, but are entirely contained within r5. (G,G') r3 into r3 (+). (H,H') r4 into r4 (++). (I) r2 into r2 (++). (J,J') r2 into r4 (++). Part of the graft lying in the floor plate has dispersed rostrocaudally and cells have dispersed mediolaterally within r4. (K,K') r4 into r2 (++).

the extent of mixing between self/self, alternate pairs or adjacent segments. All the scores in the range 1-4 for individual embryos in the three categories were placed in a table. The result was that the null hypothesis was rejected with $P \leq 0.001$, demonstrating that there were significant differences in the extent of cell mixing between the three categories.



In 7 of the 91 grafts analysed, a morphological boundary was judged to have formed around all or part of the graft. In 6 of these cases the graft was of an odd/even or even/odd rhombomere combination, consistent with our previous study (Guthrie and Lumsden, 1991). In all cases, the grafted piece of tissue was larger than the average size, suggesting that a critical number of cells is required for boundary formation to take place. In one graft of r3 to r2 (Fig. 4A,A'), a curved boundary was formed between the fluorescent graft and the surrounding cells. In a few cases, a graft was inserted across a host rhombomere boundary, eg. Fig. 4E, showing a graft of r2 to r3/r4. The fluorescent r2 cells have dispersed into r4 but have formed a boundary with r3. The boundary is in a straight line, presumably reflecting the original shape of the grafted piece. In the majority of cases where very limited graft cell dispersal was recorded, no morphological

boundaries were formed. Examples ranged from completely coherent grafts, placed in the – – category (Fig. 4B,B'), to those showing limited dispersal, placed in the – category (Fig. 4D,D').

Examples of self/self, odd/odd or even/even grafts, all of which fall into the + or ++ categories, are shown in Fig. 4F-K'. The varying extent of cell dispersal is evident; for example Fig. 4F,F' shows an r3 to r5 graft (+) in which dispersal has occurred quite extensively in the mediolateral direction. In contrast, Fig. 4K,K' shows an r4 into r2 graft (++), demonstrating extensive migration of the grafted cells throughout the host rhombomere. Where part of the grafted piece became included in the floor plate, extensive rostrocaudal migration of fluorescent cells often took place (Fig. 4J,J'). Just as in odd-numbered graft combinations, extensive mediolateral movement of cells was a common feature of even-numbered grafts.

DISCUSSION

Selective adhesion is a fundamental mechanism in embryonic development. Since adhesion is a prerequisite of multicellularity, it is likely that variations in adhesion arising between different cells and tissues have been recruited and stabilised to play an important role in many morphogenetic processes. We propose that the generation of discrete but contiguous blocks of tissue that manifest qualitatively and/or quantitatively different adhesive properties is one of the primary events in the creation of the rhombomeric pattern in the hindbrain.

The main conclusion of the present study is that absence of boundary formation between two populations of rhombomere cells is reflected in a high degree of cell miscibility at the interface. Two populations whose apposition leads to boundary formation, however, tend not to mix, and segregate or remain as coherent groups rather than disperse. Small grafts of rhombomere cells in the midst of cells from an adjacent segment did not usually form a boundary with the surrounding tissue. On the rare occasions when a partial or complete boundary did form, the graft was larger than average, suggesting that a critical mass of tissue is necessary for the expression of this morphology.

Our second major conclusion is that there is a hierarchy of miscibility and thus possibly of adhesion among segmental populations of hindbrain cells at a more subtle level than can simply be equated with the ability or inability to form a boundary. For example, cells from rhombomere 3 never formed a boundary when apposed with rhombomere 3 cells or with rhombomere 5 cells (Guthrie and Lumsden, 1991). However, in both chick/chick and chick/quail grafting experiments, r3 cells showed a greater tendency to intermingle with r3 cells than with r5 cells.

Based on our earlier rhombomere transplantation study, we could conclude nothing about the cell behaviour at boundary interfaces, since we did not mark and track cells. It is interesting that some commentators assumed lack of cell mixing to be implicit in our boundary regeneration data. Indeed, previous studies of regeneration of the segment boundary in insects point to this conclusion. Observation of boundary regeneration between cells of two different colour phenotypes in *Oncopeltus* showed that the cell types segregated out along a jagged interface that gradually resolved into a straight line (Wright and Lawrence, 1981), without cells ever trespassing inappropriately across it. In r3:4 quail/chick chimaeras, the restriction of cells to one side of the boundary was almost complete. A few cells were sometimes observed in the marginal zone, having crossed the boundary, perhaps during an early phase of boundary reconstruction.

In r3:5 and r3:3 apposition experiments, chick and quail cells mixed quite extensively with one another, especially in the mantle zone, a phenomenon prominent in other chick/quail chimaeras containing neuroepithelial grafts (Tan and Le Douarin, 1991). This accords with the idea that the neuroepithelium consists of two domains, apical and basal, with tangential cell movement, axonal growth and the translocation of neuronal somata occurring preferentially in the basal zone. During cell division in the neuroepithelium, columnar cells lose their basal endfoot prior to dividing at the ventricular surface. In this dynamic environment, the major opportunity for cell movement would be expected to arise after mitosis when cells once more elongate basally. Furthermore, the ventricular surfaces of hindbrain neuroepithelial cells contain large numbers of junctional complexes, which might impede cell rearrangement in this region (Tuckett and Morriss-Kay, 1985), while basal regions are devoid of such complexes and exhibit large intercellular spaces, particularly in the boundaries.

Grafts of rhombomere cells into the adjacent territory yielded little cell dispersal in three out of four graft types. The fourth case, r3 into r4 grafts, dispersed in the majority of embryos, raising the possibility of non-reciprocity, since when r4 cells were grafted into r3, the majority of grafts remained segregated. It should be noted that, of the 27 embryos analysed with grafts into the adjacent rhombomere, only 11 fell into the – – category. The variability of results in a single category may also be related to the variability in size of the grafted piece; the formation of boundaries around larger grafts suggested that the manifestation of cell properties is modified by the size of the group.

Despite some variability of results within and between categories, clear trends did emerge. Orthotopic graft categories scored uniformly highly in our 'dispersal index', as did the r4 into r2 even-numbered combination. Odd-numbered combination grafts scored lower than orthotopic grafts, consistent with the results obtained using chick/quail chimaeras. The reduced dispersal of quail r5 cells into chick r3 territory, when compared to dispersal of quail r3 cells into chick r3, is consistent with the higher dispersal index of chick r3/3 combinations with respect to r3/5 combinations. Grafts of odd-numbered into even-numbered rhombomeres and vice versa scored low. The differing values of the dispersal index for the various graft combinations are consistent with the idea that cells in self/self grafts mix more extensively than those from alternate segments, which in turn mix more freely than combinations of cells from adjacent segments. This is compatible with a scheme of adhesive hierarchy whereby individual rhombomeres express different repertoires of cell surface molecules. In this scheme, r3 cells would be similar but not identical to r5 cells, and relatively more different from r2 and r4.

Several factors allow us to reconcile the apparently incomplete restriction of cell mixing that we have observed with the all-or-none restriction of compartments seen in single cell marking experiments (Fraser et al., 1990). Transplantation experiments constitute a less rigorous analysis and, while single cell labelling asks questions about cell behaviour at the time boundaries are being set up, the present study requires either that the cells reiterate part of their developmental programme, or that their programme at the completion of segmentation is stable. Transplantation experiments were carried out at stage 10-12, while lineage analysis spanned stages 6-12 (Fraser et al., 1990), and both experiments were analysed at approximately the same developmental stage (19-21). Unlike single cell labelling, grafting required a recovery period, while the mean interval for cell dispersal was shorter. Thus we might expect the mean distance moved within the neuroepithelium by the progeny of injected cells to be somewhat further than by grafted cells in the present study. However, a comparison shows that the two sets of data do not differ greatly (considering only the rhombomeric dimensions in a stage 19 embryo, and ignoring growth). Clones of lineage marked cells that did not abut a boundary tended to expand isometrically to a mean diameter of 88 μm. Thus an estimate of the distance moved by a single cell might be in the region of 44 μm. In quail/chick grafts, the maximum distance moved by cells in r3/3 combinations was 34.5 μm, not significantly less. In chick/chick grafts, the edge of the grafted tissue was difficult to define, but in grafts of r4 to r4 showing maximal dispersal, cells had moved about 60% of the mediolateral extent of a rhombomere (80 μm).

Selective adhesion is a classic phenomenon first documented for the species-specific sorting of sponge cells (Wilson, 1907). Later studies showed that cell type specificity prevailed over species specificity, since in reaggregation assays using organ rudiments, chick kidney cells would aggregate with mouse kidney cells, but would segregate out from chick chondrocytes (Moscona, 1951, 1956). That this preferential adhesion of cell types was also a feature of early embryonic development was demonstrated by Townes and Holtfreter (1955), who found that disaggregated amphibian cells from the three germ layers would reaggregate and sort out in roughly the same configuration as in the embryo, i.e. endoderm inside and ectoderm outside with mesoderm between. The model of selective adhesion as a primary process in embryogenesis was later modified by the idea of temporal specificity, suggesting that the cell properties necessary for selective adhesion exhibit stage-specificity in embryogenesis (Curtis, 1961). A parallel with the hindbrain might be suggested by the fact that the maturation of even-numbered rhombomeres takes place ahead of that of odd-numbered rhombomeres.

The concept that sorting out behaviour might be attributable to quantitative differences in the strengths of intercellular adhesion was developed by Steinberg (1963, 1970). The final configuration adopted by a mixed cell population was envisaged as reflecting the minimisation of total adhesive free energy, with the less adhesive cell type coming to envelop the more adhesive cell type. Experiments with embryonic chick tissues in which cell types were either dissociated and reaggregated or confronted as tissue fragments showed that for various tissue pairs, there was a hierarchy of preference for the internal position. In some cases the internal population sorted out only very slowly, with small cell groups coalescing into larger groups but failing to form a single aggregate (Steinberg, 1970). In some cases, this situation was observed when the internal cell type was at a much lower concentration than the external one (Trinkaus and Lentz, 1964). This incompletely sorted configuration applied to pairs of tissues that had very similar positions in the adhesive hierarchy.

In attempting to draw comparisons between these studies and cell dispersal in the hindbrain, it has to be borne in mind that the constraints on cell movement differ from those in spherical aggregates or from cells cultured on planar substrata. Nevertheless, we can presume that the tendency of grafted tissue pieces to remain coherent reflects levels of adhesion between the grafted cells different from those between grafted and host cells. In the case of grafted cells that had dispersed singly or in small clusters it might be predicted that their adhesive affinities for each other are similar or identical to their affinity for the host cells. Work on *Drosophila* has shown that imaginal disc cells bind preferentially to the epidermis of the embryonic segments from which they are derived (Gauger et al., 1985), while cells from different regions of the *Xenopus* neural plate, destined to contribute to discrete parts of the nervous system, exhibit sorting behaviour in culture (Jacobson, 1980; Jacobson and Klein, 1985).

Segmentation is a common theme in development, yet mechanisms by which segments are generated vary widely. In short germ band insects, a small posterior region gradually elongates, sequentially producing segments. Conversely, in long germ band insects, such as *Drosophila*, almost the entire blastoderm is simultaneously segmented. It seems probable that the mechanisms of segmentation in long germ band insects and in the higher vertebrate hindbrain share certain properties. In both cases the pattern of segmentation is prefaced by expression of defined subsets of transcription factors, presumably leading to selective activation of cell adhesion molecules and hence to segment generation.

The transcription factors involved in setting up the early pre-segmental fields in *Drosophila* have been well characterised (Ingham, 1988). Individual rhombomeres also express unique combinations of transcription factors. The *Krox-20* gene is expressed only in r3 and r5 (Wilkinson et al., 1989a), r5 but not r3 expresses *Hoxb-3* (*Hox 2.7*) (Wilkinson et al., 1989b) and r4 only expresses *Hoxb-1* (*Hox 2.9*) (Frohman et al., 1990). These transcription factors must then act directly or indirectly on genes involved in setting up segmental patterns, such as those encoding adhesion molecules. Recent work in *Drosophila* has supported this prediction, showing that homeobox-containing genes directly regulate expression of cell and matrix adhesion molecules. The *connectin* gene, which encodes a homophilic cell adhesion molecule (Nose et al., 1992), is a target for transcriptional regulation by the homeobox gene *Ultrabithorax* (Gould et al., 1990). *Hoxb-5* (*Hox 2.5*) and *Hoxb-6* (*Hox 2.4*) respectively up-regulate and down-regulate transcription of *N-CAM*, and *Evx-1* upregulates transcription of the morphoregulatory extracellular matrix molecule cyto-

tactin (Jones et al., 1992a,b). If, as is likely, a similar network of interactions is occurring in the hindbrain, our proposed scheme of differential miscibility would be the consequence of the expression in each rhombomere of an unique combination of transcription factor-encoding genes.

While the segments in *Drosophila* form simultaneously, however, rhombomere boundaries appear sequentially (Vaage, 1969). A scenario could be envisaged in which the early establishment of broad fields in the hindbrain would generate regions of non-mixing cells and hence the first rhombomere boundaries. Subdivision of these regions in response to new or more refined expression patterns of transcription factors would eventually produce rhombomere-sized segments. This hypothesis is consistent with the described narrowing of the expression domains of *Krox-20* (Wilkinson et al., 1989a) and Hox genes (Wilkinson and Krumlauf, 1990) during stages immediately prior to boundary formation.

In order to examine the molecular basis of cell sorting among hindbrain segments, it will be necessary to adopt a more reductionist approach, by mixing marked cells from different rhombomeres in aggregation cultures. Any cellular segregation that is observed can then be perturbed using panels of antibodies against cell surface antigens with proven or suspected roles in cell adhesion and recognition.

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